of stress-inducing factors in the promotion of hypertension, which is followed by cardiovascular and other lesions.

The experimental models to be employed in these studies will be designed to effectively control or to introduce stress, ranging from a completely serene situation to a rigorous psychosocial environment that is known to produce hypertension and cardiovascular lesions, and simulates in a controlled way some of the varieties of stress experienced by man in his present society.

Various physical, biological, and chemical factors will be superimposed upon this controlled environment in order to examine their various individual influences, together with possible synergistic effects of stress in combination with chemical and other environmental factors.

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competance of the host and thus upon cancer and many other diseases.

We have now shown, for the first time to our knowledge, that stressful events normally encountered in the routine shipping, housing, and handling of mice, produce remarkable elevations in plasma corticosterone which are accompanied by equally rapid reductions in circulating lymphocytes, and an involution of the thymus (6). Thus physiologically-derived elevations of cortisosterone are capable of producing the results observed heretofor only following injections of rather large doses of corticosteroids. The above results are shown in the following figures.

Figure 1 demonstrates some of the tangible biochemical consequences of inadvertant stress during and following the routine porcedure of shipping mice from the commercial breeder's establishment to the laboratory. Upon receipt of the mice, it may be seen that the circulating corticosterone is elevated about 5-fold above normal (all samples taken at 1:00 P.M.). As shown, this physiological elevation was sufficient to produce a corresponding involution of the thymus. A significant decrease in circulating lymphocytes (T-cells) was also observed. After the mice are placed in a reassuring, uncrowded environment, the measurable effects of the shipping stress gradually subside. Corticosterone and lymphocyte levels are back to normal within a few days although a normal thymus weight is not regained until 1 1/2 to 2 weeks.

Figure 2 demonstrates further that the routine handling of experimental animals can have a striking influence on physiological parameters that may relate to hypertension. As shown here, the excitement induced in mice during the moving, capturing, and handling which accompanies routine orbital bleeding and other laboratory procedures, produces an alarm reaction with an increase in plasma corticosterone. A corresponding rapid loss in circulating lymphocytes (down to 50% after 40 minutes) and a loss in thymus weight were also observed. The cluster of disconnected points at the bottom of the curve in Figure 2 show that if ten mice in a given cage are bled rapidly, within 3 1/2 minutes, the blood samples show no evidence of corticosterone elevation. However, if the blood samples are obtained from similarly disturbed mice at longer, spaced intervals, a rising curve exhibiting the steadily increasing corticosterone concentration in the blood, beginning at 4 minutes, is obtained.

It is important to note that these corticosteroid increases are physiologically produced and thus can be considered a "normal" range that the host is repeatedly exposed to during experimental manipulation, and handling during observation.

AIMS AND OBJECTIVES

In the studies proposed in this application, we expect to gain a greater understanding of the biochemical and biological mechanisms whereby psychosocial stress leads to hypertension, atherosclerosis, and other cardiovascular diseases. Following are some of the studies which we propose to carry out.

1. Establish non-stress baseline values for adrenal corticosteroids as a function of mouse strain and sex.

Some of this information has already been established in our laboratories. We have determined the diurnal variations in BAF_1 female mice. Still needed are similar determinations in male BAF_1 mice and in other strains of mice. A micro fluorimetric assay procedure for plasma

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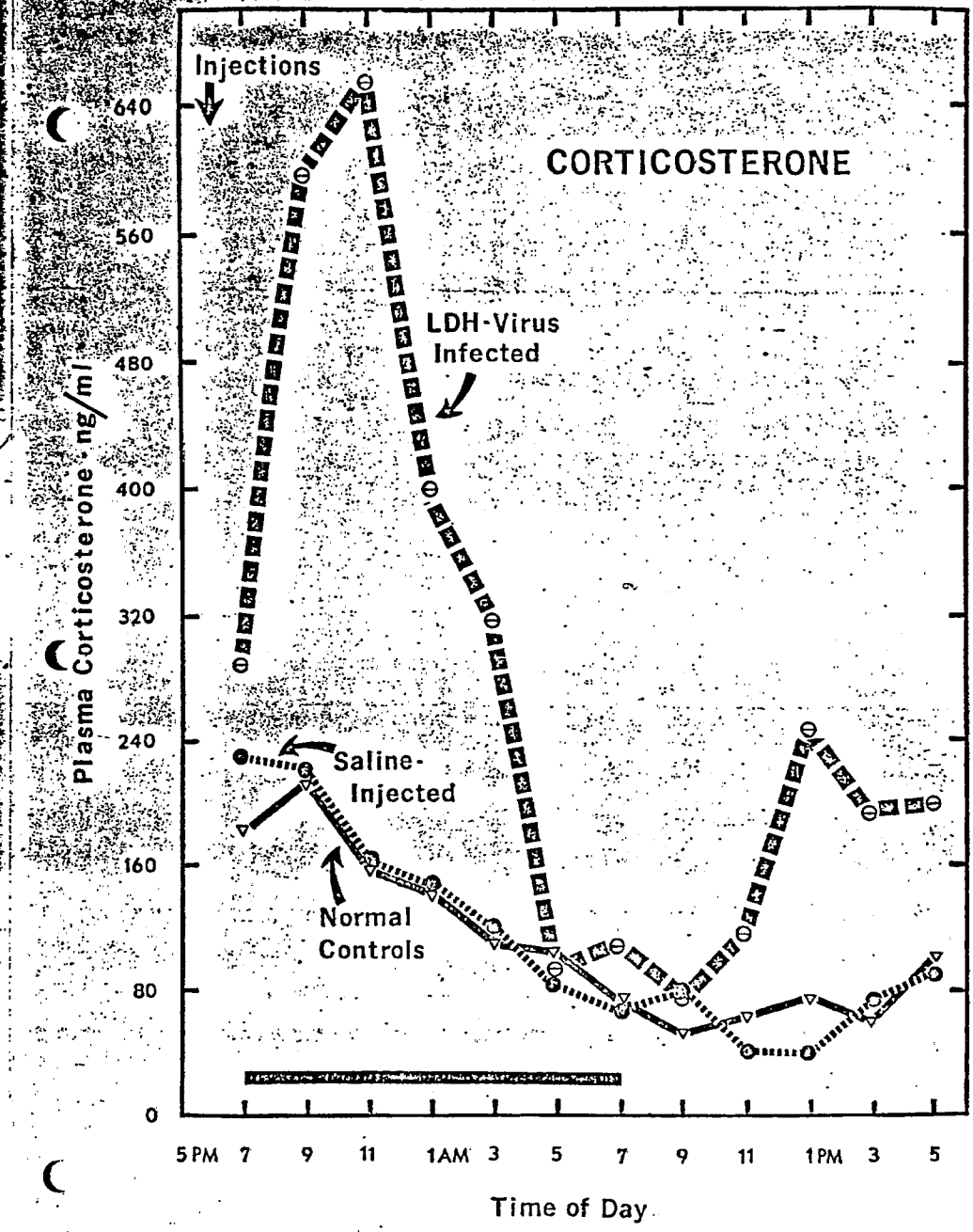

Fig. 3

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a) How do pertinent dietary alterations influence the onset, progression, control, and possible reversal of atherosclerosis?

b) Having established the influence of controlled social stress upon hypertension and the pathological sequalae, what are the synergistic influences of adding the effects of tobacco smoke, tar, CO, NO_2, nicotine, caffeine, etc., upon the usual course of cardiovascular disease? It may be noted that the population cage complex can be designed for gas and aerosol intrusion without introducing the concomitant factor of stress associated with abnormal confinment or restraint.

c) Is there a synergistic role of benign or pathogenic viruses and other infectious agents in promoting atherosclerotic and cardiovascular and renal pathologies?

Current studies in our laboratories have demonstrated the capabilities of a benign virus for inducing plasma corticosterone elevation, lymphocytopenia and thymus involution. These influences appear to be indirectly caused by physiological stress by way of the pituitary-adrenal-corticosteroid pathways, rather than by a direct viral attack upon thymus tissue. Inasmuch as such physiological effects are also incriminated as etiological factors associated with the experimental psychosocial stress-mediated hypertension, renal lesions, and cardiovascular pathologies observed in mice, and presumed to function in human beings, it would be appropriate to examine the possible role of such infectious agents in this category of disease. Figure 3 illustrates our observations of the induction of high levels of plasma corticosterone following the intentional infection of mice with a benign, ubiquitously distributed murine agent, the LDH-elevating virus.

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This reagent is mixed fresh daily.

Procedure. Aliquots of 50 µl of plasma or working standards of corticosterone (or cortisol) are pipetted into reaction flasks followed by 50 µl of water. Volumes of 650 µl of chloroform are then added to each flask from a repetitive pipette dispenser. The flask is stoppered and mixed on a vortex mixer (in groups of four) for 60 seconds to extract the corticosterone. After centrifugation (1 min) and removal of the aqueous layer by controlled vacuum aspiration with a glass capillary, 100 µl of 0.1 N NaOH is added, the flasks vortexed for 15 seconds, centrifuged, and the aqueous layer again removed. An aliquot of 500 µl of the chloroform solution is then transferred to another reaction flask containing 400 µl of the acid-alcohol reagent (prepipetted before the extraction procedure is started). After 30 seconds of vortex mixing, followed by centrifugation, the upper chloroform layer is removed by aspiration and the total acid-alcohol volume transferred to a 6 x 50 mm Pyrex culture tube cuvette. The fluorescence is allowed to develop at room temperature (22 to 23°). Readings are taken at 80 min and again at 90 min, starting from the time when the chloroform solution is mixed with the acid-alcohol reagent (in a Turner Model 111 fluorometer fitted with a micro cuvette adaptor). A general purpose lamp (#110-850) plus Turner 2A and 47B filters provide a 436 nm excitation beam. For the emission wavelength a 2A-12 filter is used.

Calculations. We have determined that a fluorescence reading (corrected for blank) that is linear with concentration is obtained when samples of corticosterone varying from 50 to 800 ng/ml are assayed by this procedure. Therefore fluorescence units from plasma samples are converted to concentration units by multiplying by the constant derived from the foregoing relationship. In addition, the standardization is constantly monitored by analyzing duplicate samples of corticosterone standard with each 12-sample batch that is processed. Two of the samples are water blanks which are then used to correct the other sample values.

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THE COUNCIL FOR TOBACCO RESEARCH--U.S.A., Inc.

110 East 59th Street
New York, N.Y. 10022
(212) 421-8885

Application for Research Grant Date: 5-22-74

RECEIVED
MAY 27 1974

1. Principal Investigators:

Dr. Vernon Riley, D.Sc., Chairman, Department of Microbiology
Member, Fred Hutchinson Cancer Research Center.

Dr. George A. Santisteban, Ph.D., Senior Biologist, Department of Microbiology.
Professor of Biology, Seattle University.
Affiliate, Fred Hutchinson Cancer Research Center

Dr. Darrel H. Spackman, Ph.D. Senior Research Biochemist, Department of Microbiology.

2. Institution & Adress:

PACIFIC NORTHWEST RESEARCH FOUNDATION
1102 Columbia Street
Seattle, Washington 98104

3. Departments where research will be done or collaboration provided:

Department of Microbiology, PNRF
Department of Biology, Seattle University

4. Short title of study:

A SMALL ANIMAL MODEL FOR THE STUDY OF FACTORS ASSOCIATED WITH HYPERTENSION AND CARDIOVASCULAR DISEASE.

5. Proposed starting date: June 1st, 1974.

6. Estimated time to complete: Two years.

7. Brief description of specific research airms:

A small animal model will be utilized to investigate the etiological role of a variety of experimental parameters in the induction of essential hypertension, renal lesions, atherosclerosis, and other manifestations of cardiovascular disease.

An important aspect of the working hypothesis of this project will be the strict control of environmental factors. This can be accomplished in a practical way only through the use of small animals, preferably genetically defined mice.

Information presently available, through data produced by our laboratory and others, demonstrates the vital role of various forms

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In analyzing tissues for free and non-protein bound amino acids, one significant problem is what to do with glutathione. Although absent in plasma and urine, both the reduced (GSH) and the oxidized (GSSG) forms are present in various tissues and red blood cells. They chromatograph as separate peaks, and interfere with the chromatography of other amino acids. The GSSG peak is broad and overlaps several amino acids using either the sodium citrate buffer system (25, 44) or the lithium citrate system (D.H. Spackman, per. obser.) for chromatography of acidic and neutral amino acids. In the lithium buffer system, used for our analyses, GSH partially overlaps aspartic acid. Although GSH is normally absent from mouse plasma, any hemolysis of red cells (which contain GSH) before separation of the plasma will introduce this peptide into the plasma fraction. This is potentially serious in asparaginase therapy studies where accurate measurements of aspartic acid are needed. An effective method has been described (25, 44) for removing GSH and GSSG by conversion to the sulfonate, but this procedure is rather time-consuming and thus does not readily lend itself to schedules involving the preparation of multiple samples. Preliminary experiments have been undertaken and it is expected that we will be able to accelerate and improve this aspect of sample preparation.

Very few studies have been made to systematically investigate the detrimental effects of delays or of various procedures upon the integrity of the sample to be analyzed. Neither have critical studies been completed to determine the effects of both short-term and long-term storage of samples awaiting analysis. One report has shown that both asparagine and glutamine decrease in concentration following storage of frozen, non-deproteinized plasma samples, and that cystine is completely lost after seven months storage of such samples (38). Our own results show that most of the cystine is lost from plasma samples in as little as 2 or 3 days of storage at 4°. If asparaginase is present in the plasma, one hour of storage at 4°, or 24 hours at -20° will cause severe losses (50 to 95%) of both asparagine and glutamine.

There is little doubt that the amino acids in deproteinized samples are much more stable than they are in plasma. A few analyses have been made in our laboratories to determine time-course changes in the acidic and neutral amino acids in samples prepared from mouse plasma. These samples were analyzed when freshly prepared and again after 21 months of frozen storage (at -20° for 12 months and -80° for 9 months). The average changes from four such samples are shown in Table IV. These data show that while most amino acids are quite stable, some changes do take place. The greatest percentage change of those listed is for asparagine.

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3. Determine the changes in plasma and tissue amino acids produced by elevations of adrenal corticosterone. These amino acid studies would be carried out following experimental injections of corticosteroids as well as a part of long-term psychosocial, hypertension studies using the population study cages.

Adrenal corticosteroids have several specific influences on carbohydrate and amino acid metabolism, including a gluconeogenesis from amino acids. The latter may result from an increased availability of amino acids because of the inhibiting effect of adrenal cortical steroids on amino acid incorporation into proteins. Specifically, protein synthesis is inhibited in muscle and other tissues at high hormonal levels. Presumably, adequately high levels would be obtained under stress. Since protein degradation in the same tissues is continuously occurring, there is a net loss of amino acids resulting in tissue wasting. Metabolic oxidation of these amino acids would thus lead to an overall negative nitrogen balance. Under such conditions plasma free amino acids would be expected to be elevated.

The foregoing mechanisms may explain the observations made by Henry, et.al. (2) that in psychosocial stress there seems to be a more rapid loss or wasting of body proteins (thin skin, etc.). Such mechanisms may also be associated with the elevated blood pressure and obesity observed with Cushings Syndrome (glucocortcoid elevation) (11), and Conn's Syndrome (aldosterone elevation) (12).

4. Determine whether any cortisol, in addition to the predominant corticosterone, is present in mouse plasma, either normally or as a result of severe chronic stress.

It has been established that the only circulating 17-hydroxycorticosteroid in the rat is corticosterone (20). Cortisol apparently is not present. We intend to make such a determination in the mouse. This information is of some importance, for on the one hand, cortisol gives only one-fourth the fluorescence in assay procedures as does corticosterone, but on the other hand, cortisol is many times more active than corticosterone in most of its physiological effects, including thymus involution. Thus small amounts of cortisol would be difficult to detect during assay and yet, if present, could account for a significant part of the total physiological effects observed in association with stress.

5. Initiate studies to answer the question as to whether the control of diet can retard or prevent the progressive hypertensive and cardiovascular changes resulting from psychosicial stimulation and stress.

Our plasma and tissue amino acid studies may give valuable clues in this direction. For example, if certain essential amino acids were to be found in low concentration, dietary supplementation could perhaps improve overall protein formation and thus improve the overall general health of the host. Ratios of essential to non-essential amino acids also can be a useful index of general nutrition (21).

6. Following the establishment of optimal conditions for measuring the various parameters associated with progressive development of hypertension and cardiovascular and renal lesions, this small animal model and its physical devices may be employed in a variety of experiments to examine the following questions.

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Riley, V., Spackman, D.H., Fitzmaurice, M.A., Roberts, J., Holcenberg, J.S., and Dolowy, W.C.: Therapeutic properties of a new glutaminase-asparaginase preparation and the influence of the LDH-virus. Cancer Res. 34:429-438 (1974).

In addition, the following manuscripts have been submitted or are being prepared and will be submitted for publication in the near future:

Riley, V., and Spackman, D.H.: Theoretical and practical considerations in the use of hemodialysis for the therapeutic removal of asparagine and other amino acids. (Manuscript Submitted).

Riley, V., Spackman, D.H., Fitzmaurice, M.A., Roberts, J., Holcenberg, J.S., and Dolowy, W.C.: Alterations in plasma amino acids by a glutaminase-asparaginase preparation and the influence of the LDH-virus. (Manuscript in Preparation).

Riley, V., Spackman, D., and Fitzmaurice, M.A.: Asparaginase: An experimental and clinical model for new concepts in cancer research. CRC Critical Reviews. (Manuscript in Preparation).

Spackman, D.H.: Amino acid analysis of physiological samples with a high resolution, high sensitivity system. (Manuscript in Preparation).

Spackman, D.H., and Riley, V.: Free amino acids in the plasma, urine, and tissues of mice: Effects of sample preparation, fasting, and tumor growth. (Manuscript in Preparation).

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corticosterone was developed in our laboratories specifically for use with the small samples of blood which can be obtained serially from mice by using the rapid and convenient orbital bleeding procedure.

2. Expand the psychosocial stress studies initiated in our laboratories by Dr. Sanisteban (2, 3), using specially designed population study cages, and correlating such "normal" stress factors with progressive essential hypertension and arteriosclerotic changes in the heart, kidney and aorta.

Our search for an explanation of the mechanisms responsible for the above phenomena has led us into consideration and examination of the hormonal and neurobiological aspects of the defense alarm responses. Evidence of increased activation of the sympathetic nervous system and both components of the adrenal gland has been obtained. Thus in our chronically socialized mice there have been shown to occur increases in blood levels of catecholamine biosynthetic enzymes, epinephrine and norepinephrine (2, 7). Also, we have observed involution of the thymus and depletion of adrenal cholesterol and ascorbic acid (8). Preliminary results also show a definate elevation in plasma levels of corticosteroids during social stress.

These results are in accord with human studies which have shown that persons undergoing typical defense alarm stimulation have increased levels of 17-hydroxycorticosteroids (17-OHCS) (9); whereas a fall in 17-OHCS levels occurs in response to soothing, relaxing inputs (10). Clinical conditions characterized by high blood pressure and high levels of 17-OHCS include Cushing's Syndrome, in which there is an excessive production of glucocorticoids (11), and Conn's Syndrome, where there is a high blood level of aldosterone (12). Treatment of adrenal cortical deficient patients with large amounts of 17-OHCS leads to an increase in blood pressure. Conversely a drop in blood pressure is characteristically observed in Addison's disease and following adrenalectomy (13). Finally, hypertrophy of the adrenal cortex is often observed in animals undergoing chronic stimulation (14). Thus it appears that the pituitary-adrenal cortical system is intricately involved in the induction of hypertension by acute and chronic social stress.

Our studies on the sequence of pathological changes occurring in the heart, blood vessels, and kidney in socially stimulated hypertensive mice (2), support the reports that at high blood pressures the walls of the small arteries deteriorate and tissue nutrition fails due to a developing obstruction in blood supply (15, 16). In the heart, the vascular damage impairs the blood supply to the myocardium, which, with the progressive elevation in blood pressure, has a progressively increasing work load. In the brain there results an ever-increasing risk of death or disablement from brain hemorrhage. In the kidney the vascular derangement leads to the development of glomerulonephritis and, less frequently, to pyelonephritis (17, 18). With increasing obstruction, there is a progressive elevation of blood pressure. If the rise is rapid and severe, it will be associated with an increased renin production and the resulting elevation in blood pressure can lead to levels in excess of 200 mm Hg in a matter of weeks. This can be fatal within a few months unless it is controlled by surgery or by the use of drugs (17, 18, 19).

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DISPOSABLE FILTER DETAIL AT AIR INTAKE END OF SHELVES
FILTERS IN POSITION AT AIR INTAKE END OF ENCLOSED SHELVES
AIR CONTROL LOUVERS AT EXHAUST END OF SHELVES
ENCLOSED SHELVES READY TO RECEIVE MOUSE CAGES
SHELVES IN OPERATION WITH VIRUS COLOR CODE CARDS
SLIDING SHELF AND DOUBLE DOOR PASS-THRU BETWEEN CLEAN AND VIRUS ROOMS

Figure 4

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Population Study Cage for the Study of Psychosocial Stress. This consists of a system of interconnected Lucite boxes which allows free intercommunication but also forces the animals to come into contact with each other in a central area which thus becomes a "behavioral sink", as described by Calhoun (22). In the central area there is a hexagonal pen topped with a quarter-inch wire mesh cover from which are suspended a feeding basket and tubes from two watering bottles. Six plastic runways (1 1/2" X 6") radiate from each side of the pen, each leading into a standard "shoe box" type cage. Each box is covered by a removable quarter-inch wire mesh lid. There are one and one half inch plastic tubes interconnecting all adjacent boxes. In this system the animals may move from box to box by way of the peripheral runways and into the central pen from any of the peripheral pens. Studies of the behavioral and adrenocortical and blood pressure responses of animals kept in this life system show that the animals in these pens develop a sustained hypertension (2), and an elevated adrenocortical stimulation (8). These responses are particularly marked in males. It has already been shown that animals raised in isolation are more sensitive and more likely to develop arterial hypertension when placed in this apparently socially stressful situation. The construction of a population study cage is shown in Figure 5.

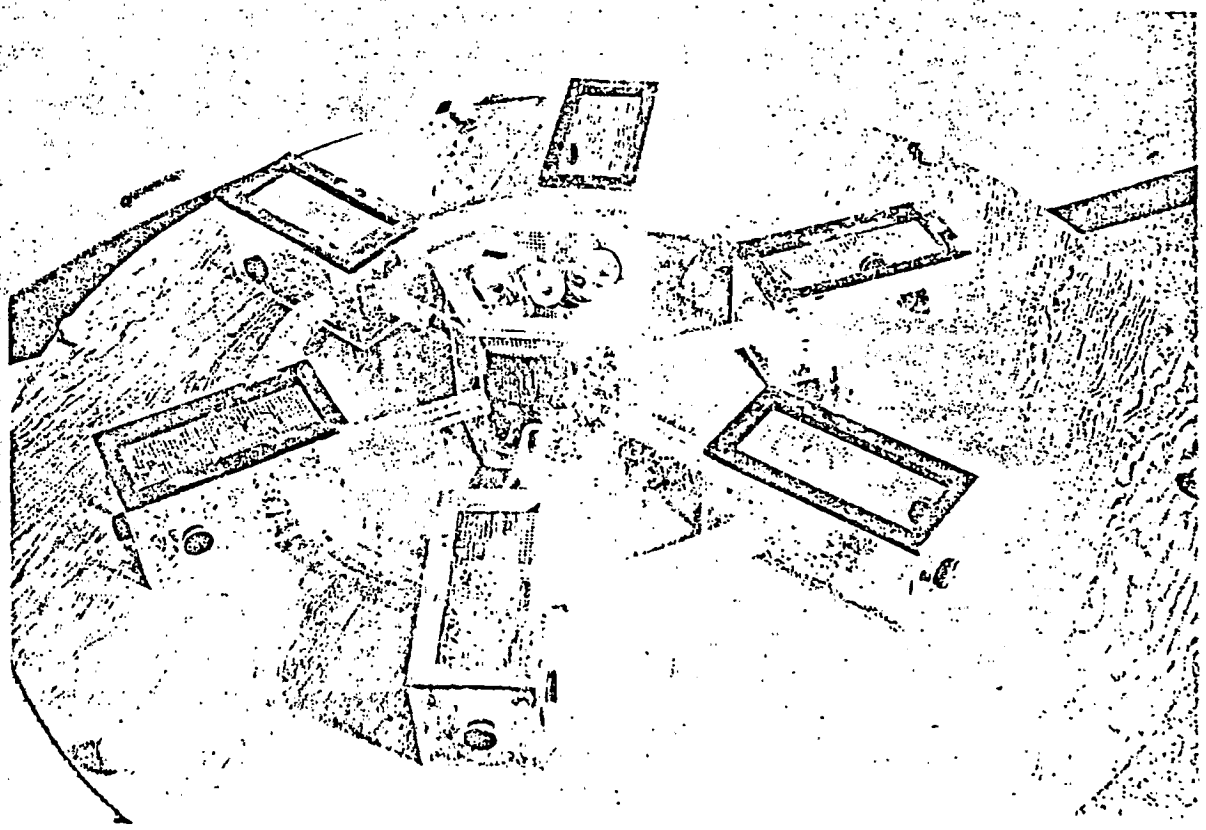

FIGURE 5

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R: REDACTED MATERIAL

14. PROVISIONAL BUDGET FOR THE FIRST 12-MONTH PERIOD

SALARIES		Salary	F/B	Total
Dr. Vernon Riley	Co-Principal Investigator			
Dr. George Santisteban	Co-Principal Investigator			
Dr. Daniel Spackman	Co-Principal Investigator			
D. Lucille Bredberg	Research Associate	REDACTED		REDACTED
To be hired	Research Assistant			
	Total			

Note: All salaried personnel will contribute 100% of their time to this project. The non-salaried co-principal investigators will contribute their time as required by the project.

CONSUMABLE SUPPLIES

Mice, food, bedding, disposable syringes, needles bleeding tubes, miscellaneous glassware, reagents, special supplies for the amino acid analyzer and the Coulter Counter, and the florescent spectrophotometer, miscellaneous supplies and minor items of equipment. $ 6,000

SPECIAL SERVICES

Medical illustrations, photography, slide preparations, reprints, page charges, duplication, equipment maintenance. $ 2,000

SPECIAL EQUIPMENT

Instrument for measuring blood pressure in mice. (Tail plethysmography: pressure cuff, strain gauge, Sanborn recorder.) Approx. $ 5,000

TOTAL $55,979

INDIRECT COSTS: To be negotiated.

15. ESTIMATED TIME REQUIRED TO COMPLETE THESE STUDIES:

It is obvious that all of the various experimental ramifications of these studies cannot be completed during the 12-month period of this budget. It is suggested that initial approval for a two year period be considered, with the amount for continuing support being determined by the rate at which the council feels the studies justify at the end of the first year. No special equipment is anticipated for the 2nd year.

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for amino acids and related compounds, has been the picric acid method (38). This method is objectionable, however, because of the length of time and significant manipulation required for the operation. Other widely used methods have made use of sulfosalicylic acid (SSA) (39-42) although trichloroacetic acid, phosphotungstic acid, and periodic acid have also been used for this purpose. Another method makes use of low sample pH and ultracentrifugation for a partial removal of sample proteins (40). Ultrafiltration has also been used (38, 43).

Because of the limited volume of mouse plasma available for amino acid analysis, we have developed methods which allow all of the plasma sample to be utilized in producing the final deproteinized sample. For these purposes sulfosalicylic acid has been selected as the protein-precipitating agent of choice. Sulfosalicylic acid solutions have been used at several different concentrations and total volumes for the deproteinization of physiological fluid samples. Our investigations show that significant errors can be inadvertently introduced in most of these methods due to failure to obtain total recovery of the amino acids at each step in preparing the sample. We have, therefore, developed a reproducible, effective method designed to eliminate these errors. It utilizes plasma samples of 1 ml or less and does not require volume reduction of the deproteinized sample prior to analysis on the amino acid analyzer. The pH of the sample is carefully controlled in order to give the most efficient precipitation of proteins during preparation without destruction of labile amino acids, and to give optimum, reproducible chromatographic placement of pH-sensitive amino acids during the analysis. After the initial precipitation and removal of supernatant, the remaining precipitate is suspended and extracted three additional times to give better than a 99% recovery of all free amino acids originally in the plasma sample.

Inasmuch as the circulating amino acid pool in vivo represents a host equilibrium between influx, clearance, enzymic alterations, and perhaps other physiological events, it is apparent that important changes in certain amino acids will occur in blood removed from the host if the samples are not obtained, processed, and stored under conditions designed specifically to prevent such alterations. For example, rapid changes would be expected to occur in asparagine concentration in the presence of asparaginase. Appropriate sampling procedures have, therefore, been developed to minimize amino acid alterations during both the collection of blood and the processing of plasma samples for amino acid analysis.

Essential elements of these procedures include keeping the blood samples cold during processing, reducing sample preparation time to a minimum, and precipitation of plasma proteins, including all enzymes, as rapidly as possible after the withdrawal of the blood sample.

One or more separate blood samples of approximately 0.2 ml volume are removed from each mouse by the orbital bleeding technique using disposable, heparinized bleeding tubes. The actual bleeding procedure requires only a few seconds. After filling, each tube is quickly stoppered and plunged into an ice bath, chilling the blood immediately to 0°C. The blood samples are centrifuged at 0°C, and the cold plasmas are transferred immediately into pre-weighed, disposable tubes containing the protein precipitating agent. Thus, all plasma proteins are removed from solution at the earliest possible moment and the enzyme activities are terminated.

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PROCEDURES USED WITH AMINO ACID ANALYSIS

Mice. Hybrid BAF (C56Bl/6 x A) F_1 mice will be used initially for these studies. Other strains, such as BDF_1 and C3H, are carried in our laboratories, and will be used as required. Female mice between 6 and 12 weeks of age, weighing 18 to 22 grams will be generally employed. All mice received from breeders are placed under close observation for a period of 2 weeks for signs of any respiratory or other diseases or other evidence of ill health before being used in experiments.

Blood Sampling Techniques. Inasmuch as the circulating amino acid pool *in vivo* represents a host equilibrium between influx, clearance, enzymic alterations, and perhaps other physiological events, it is apparent that losses of certain amino acids will occur in blood removed from the host if the samples are not obtained, processed, and stored under conditions designed specifically to prevent such losses. For example, rapid changes would be expected to occur in plasma asparagine concentration during the administration of asparaginase, which has a specific effect on this amino acid. Appropriate sampling procedures have, therefore, been designed to minimize amino acid alterations during bleeding and the further processing of plasma samples for amino acid analysis (50).

Essential principles of these procedures include keeping the blood samples cold during processing, reducing sample preparation time to a minimum, and precipitation of plasma proteins, including all enzymes, as rapidly as possible after the withdrawal of the blood sample.

Blood samples of about 0.2 ml per mouse are removed form 4-hour fasted mice by the orbital bleeding technique (51) using pre-chilled, disposable, heparinized bleeding tubes. The actual bleeding procedure requires only a few seconds; the tubes are then quickly stoppered and plunged into an ice bath, chilling the blood immediately to 0°C. The blood samples are centrifuged at 0°C, and the cold plasma samples are transferred immediately into pre-weighed, disposable tubes containing the protein precipitating agent. Thus all plasma proteins, including exogenous asparaginase, are removed from solutions at the earliest possible moment and all enzyme activity is terminated.

Urine and Tissue Samples. Urine samples are collected by induced voidance as needed and also in special metabolic cages for 24-hour collections. Organs and tissue samples will be removed as rapidly as possible from decapitated mice. These samples will either be homogenized, deproteinized, and further prepared for analysis immediately, or will be quick-frozen on dry ice for short periods of time until such preparation can be conveniently carried out. Healthy young adult mice will be used as a source of normal tissues, organs, and fluids. For tumor tissue, firm, non-necrotic portions of the rapidly growing tumor will, in general, be selected.

Methodological Improvements

Extensive developmental work has been carried out by the Principal Investigator in the general area of amino acid analysis. The original description of the "automatic" amino acid analyzer was published in 1958 by Spackman, Moore, and Stein (24, 25); associated methods of chromatography

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8. WORKING HYPOTHESIS

INTRODUCTION

Recent developments in the epidemiology of essential hypertension and cardiovascular disease place increasing emphasis on the etiologic role of psychosocial stimulation and stress. Some investigators assert that such psychosocial stimulation resulting in a defense alarm response constitutes a significantly more important factor than obesity, diet or exercise (1).

In work carried out in our laboratories and elsewhere (2, 3), there has been induced in an isogenic strain of mice (CBA), sustained arterial hypertension which was accompanied by degenerative changes in the myocardium, coronary blood vessels, aorta and kidneys. This resulted from subjecting the mice to prolonged and repeated social interaction in a special population cage under controlled dietary and environmental conditions. Under these conditions there also occurred an accelerated physical deterioration of the males, a reduction in the size of litters, and a failure of the females to raise their young.

The working hypothesis is that the above observations may be either directly or indirectly attributed, for the most part, to the occurrence of disproportionately elevated, prolonged and repeated defense alarm arousals, with a consequential activation of the sypathetic-adrenal medullary, and pituitary-adrenal cortical systems. Current studies in our laboratories implicate the pituitary-adrenal-thymus axis as a major factor.

The overall objectives of our proposed research are to gain a greater understanding of the biochemical and biological mechanisms whereby psychosocial stress leads to hypertension and cardiovascular disease in mice. An expanded knowledge of such physiological mechanisms in a small animal model will offer obvious advantages to subsequent studies in the human. Additional aspects of our objectives are to develop or improve the methodologies and techniques needed to establish the mouse as an optimal small animal model that may be employed to facilitate studies concerning the etiology and control of cardiovascular diseases and related pathologies.

BACKGROUND

Studies in the past have firmly established that a variety of stressful events experienced by laboratory animals or humans will produce a corresponding elevation of plasma adrenal corticosteroids. The results of controlled injections of corticosteroids has also been investigated. For example the relationship between injections of high, non-physiological doses of corticosteroids and subsequent involution of the thymus is firmly established (4). A concomitant destruction of circulating lymphocytes (T-cells) usually accompanies such thymolytic effects. In considering the question of the significance of these events upon the physiological competence of the host, such experiments have been questioned on the basis that the corticosteroid injections used to produce thymus involution have been selected to produce an approximate 50-fold elevation in plasma corticosteroid and thus far above expected physiological levels (5).

The potential importance of this question bears not only upon the etiology of hypertension and cardiovascular disease but also upon the immunological

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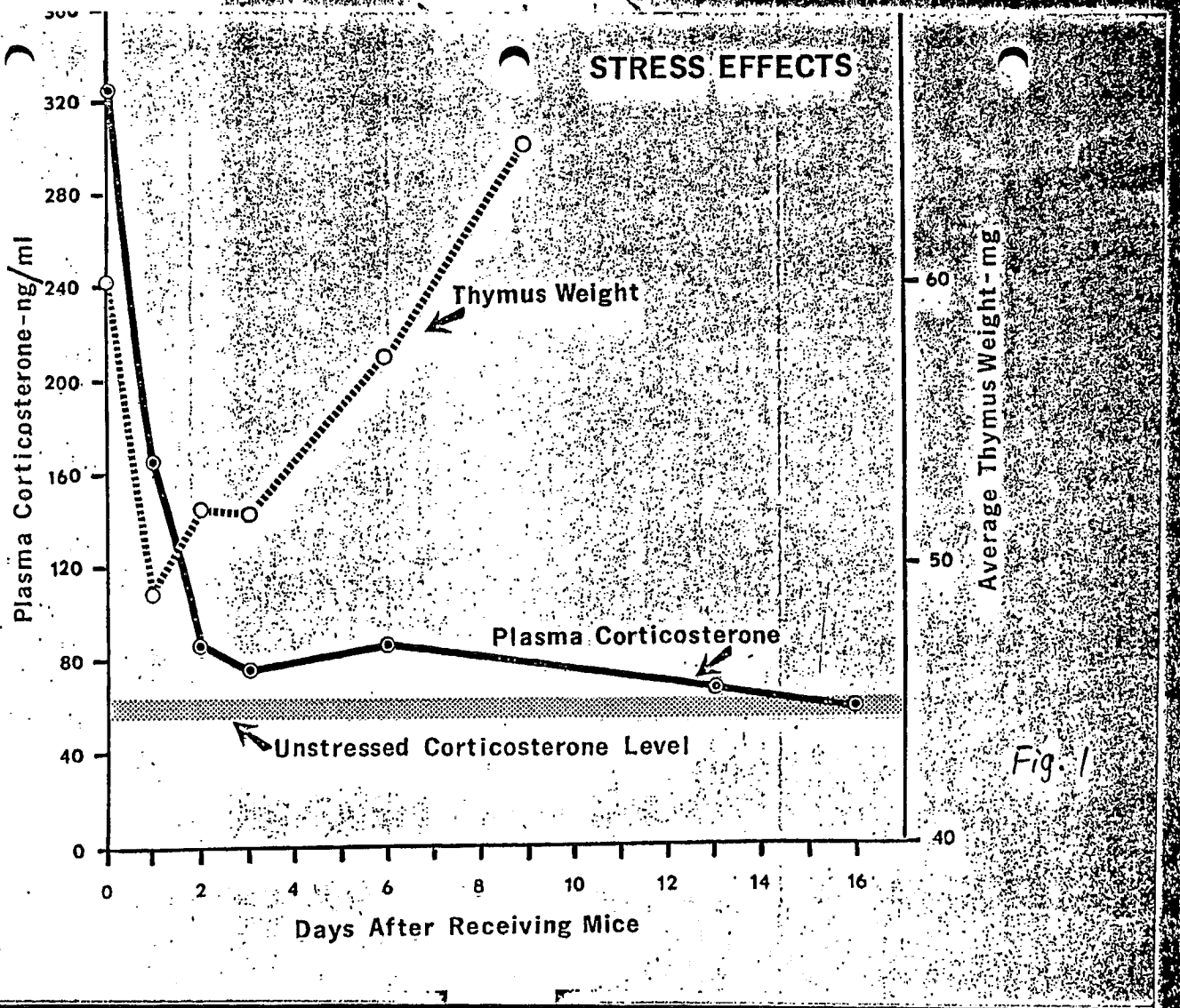

Fig. 1

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Memberships in Medical and Scientific Societies:

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12. BIOGRAPHICAL INFORMATION

(Give the following information for all professional personnel listed on page 2, beginning with the Principal Investigator. Use continuation pages and follow the same general format for each person.)

NAME	TITLE	BIRTHDATE (Mo., Day, Yr.)
RILEY, Vernon T.	Chairman, Department of Microbiology	REDACTED
PLACE OF BIRTH (City, State, Country)	**PRESENT NATIONALITY (If non-U.S. citizen, indicate kind of visa and expiration date)**	**SEX**
REDACTED	REDACTED	☒ Male ☐ Female

EDUCATION *(Begin with baccalaureate training and include postdoctoral)*

INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	SCIENTIFIC FIELD
Spokane College, Spokane, Washington	A.B.	1935	Biology
George Washington University			Biology
University of Paris (Sorbonne), France	D.Sc.	1966	Microbiology

HONORS

Doctoral Thesis, Tres Honorable, (Sorbonne); Fellow, New York Academy of Sciences; Chairman, International Pigment Cell Conferences; National Member, Society for Experimental Biology and Medicine.

MAJOR RESEARCH INTEREST	ROLE IN PROPOSED PROJECT
Basic physiological precesses involving cell biology, enzymology, and virology.	Collaborator--Department Chairman

RESEARCH SUPPORT *(See instructions)*

National Cancer Institute General Support Grant, Pacific Northwest Research Foundation, Department of Microbiology.

NCI CORE GRANT for the Fred Hutchinson Cancer Research Center.

RESEARCH AND/OR PROFESSIONAL EXPERIENCE *(Starting with present position, list training and experience relevant to area of project, including teaching and clinical responsibilities. List all or most representative publications.*

Chairman, Department of Microbiology, Pacific Northwest Research Foundation, Seattle, Washington, September 1967 to present.

Member, Fred Hutchinson Cancer Research Center, Seattle, Washington June 1973 to present

Full Professor, Experimental Animal Medicine, University of Washington School of Medicin Seattle, Washington (Affiliate), June 1971.

Head of Section, and Head of Laboratory, Division of Experimental Chemotherapy, Sloan-Kettering Institute for Cancer Research, Rye, New York, 1954-1967; Assistant, 1954-1960, Associate, 1960-1967.

Biologist, National Cancer Institute, National Institutes of Health, U.S.P.H.S., Bethesda, Maryland, 1943-1953.

Senior Research Assistant in Chemistry (Oceanographic Laboratory), University of Washington (Fermentation Research), 1937-1940.

Assistant Bacteriologist, Hollister-Steir Laboratories, Spokane, Washington 1935-1936.

The above experiences included 5 years training in quantitative virology with W. Ray Bryan (NCI), and 5 years training in enzymology and cellular metabolism with Drs. Dean Burk, Mark Woods, Jesse Greenstein, and Otto Warburg.

The principal investigator was a visiting scientist with Prof. Charles Oberling, Villejuif, France, 1959; and with Prof. Raymond Latarjet and Dr. Robert Cramer, Laboratorie Pasteur de l'Institute de Radium, Paris, 1960.

The principal investigator received his doctorate in microbiology under the Nobel laureate, Andre Lwoff, Pasteur Institute and University of Paris.

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were also described (26). Further developmental studies for increasing the sophistication of the instrument were carried out at the Spinco Division of Beckman Instruments, at the University of Washington, and at Pacific Northwest Research Foundation. Following the introduction of the automatic amino acid analyzer, improvements have been made in several ways through the efforts of several investigators. Analyses have been accelerated, thus shortening the time required for each individual assay (27-31); micro methods have been developed requiring small quantities of sample (32); and spherical-bead ion exchange resins have permitted higher flow rates and improved resolution (33, 34).

Recent improvements have been made in elution procedures utilizing lithium buffers rather than sodium buffers for the analysis of acidic and neutral amino acids (27, 35). By the use of these buffers, asparagine, for the first time, has been separated from glutamine in an overall analysis procedure.

Published procedures were found to permit only a marginal resolution of asparagine, glutamic acid, and glutamine from each other; this was also true of the resolution of serine-threonine. These procedures were also inadequate for an acceptable separation of the amino acids cystine, methionine, homocitrulline, cystathionine, isoleucine, and leucine. Homocitrulline, which is present in both urine and plasma, is completely obscured by the other amino acids in this region.

Because of these inadequacies we have developed a new, improved method of analysis using lithium citrate buffers (36, 37). A three-buffer sequence has been adopted for optimal resolution of all of the acidic and neutral amino acids normally found in physiological fluids. The resulting improved resolution of asparagine-glutamic acid-glutamine has been especially useful in our amino acid deprivation studies of leukemia using asparaginase and glutaminase.

An improved analytical method has also been devised for the chromatography of the basic amino acids found in physiological fluid samples, particularly blood plasma (36, 37). With this revised method, excellent resolution of all known basic components is achieved. A significant advantage of this new system for the basic amino acids is that it has allowed us to resolve and demonstrate the presence of a new amino acid, E-N-methyllysine, in mouse plasma (5, 11).

Appropriate changes in valves, timers, and other control equipment have been made to incorporate the above modifications into the overall, routine automatic analysis of physiological fluid samples.

An increase in sensitivity has been achieved with the Model 120B by using cuvettes which have a flow path of 20 mm in the photometer. This has been facilitated through the use of new non-darkening incandescent lamps with a tungsten-iodide filament (37).

The procedure most widely used in the past for the deproteinization of physiological fluids and tissue extracts, that are to be subsequently analyzed

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ASSAY PROCEDURE FOR PLASMA CORITOSTERONE IN MICE

Introduction. The micro fluorimetric assay procedure currently in use was developed specifically in our laboratories for use with the small samples of blood which are available from mice, and is based on the procedure described by Glick, et at (Endocrinology 74:653-655, 1964). The orbital bleeding procedure which is used exclusively in routine bleedings in our laboratory, provides 50 to 100 µl quantities of plasma. Our procedure has been designed to use 50 µl plasma samples and thus either single or duplicate assays on the blood from a single mouse may be performed as desired. The method gives accurate and dependable results using sample quantities of corticosterone that may range from 0.5 to 50 ng (10 to 1000 ng/ml). Since there is some normal biological variation in individual mice, many of our experiments utilize groups of 5 to 10 mice. The corticosterone assays are run in duplicate, triplicate, or quadruplicate on plasma pools obtained from such groups.

Washing of Glassware. This is a critical step in any fluorimetric procedure and particularly so in a micro assay. The procedures which we use have been chosen after extensive evaluation to provide thorough cleaning (and thus minimum interference) and yet permit reasonably rapid routine procedures using 12 or more assays per batch. All glassware is first rinsed or soaked in Liquinox cleaning solution. Glassware that has not been in contact with plasma or corticosteroid standards is then rinsed in tap water 10 times, deionized distilled water 3 times, and acetone (from a polyethylene wash bottle) 3 times. After air drying (not to exceed 20 minutes), the items of glassware are covered with Saran film or enclosed in containers, to prevent contamination from airborn particles and vapors. Since we have determined that "disposable" Pasteur pipetts and culture tube cuvettes must be prewashed before use, we routinely clean and use these items repeatedly. Non-heparinized bleeding tubes are washed on a neoprene adapter in lots of 24 and then heparinized with sodium heparin. The extraction tubes (Corning 2-ml volumetric flasks with glass stoppers) and other glassware which comes in contact with plasma or corticosteroid standard solutions are rinsed in Liquinox cleaning solution followed by water, drained, and are then immersed in a stainless steel basket in chromic acid cleaning solution (made by adding 70 ml of a saturated solution of sodium dichromate to two liters of technical grade sulfuric acid) a minimum of two hours but usually overnight. They are then rinsed and stored as described above.

Reagents. All organic reagents are purchased as "Reagent Grade" and are distilled before use. This includes acetone, absolute ethanol, chloroform, methylene chloride and carbon tetrachloride. Inorganic reagents are of Reagent Grade, and where water is used, it is of deionized-distilled quality.

The acid-alcohol reagent which is used to develop fluorescence, forms a critical part of the overall procedure. A 2:1 volume/volume ratio of sulfuric acid to ethanol has been found to give maximum fluorescence with corticosterone and minimum fluorescence with interfering substances found in plasma. It is mixed in a glass-stoppered Erlenmeyer flask which is partially immersed in a crushed ice bath. It is important to keep the reagent cold during mixing and subsequent storage to provide low background blanks.

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9. EXPERIMENTAL DESIGN AND PROCEDURES

General descriptions of contemplated experimental designs and procedures are included in the foregoing section, and in the attached reprints and abstracts. Following, are more detailed descriptions of some of the specific procedures that we plan to employ in carrying out the basic intent of these proposed studies. The several types of controlled experimental environments, which we have at our disposal for the study of such important daily effects upon this small animal model, are also described here in some detail. It is our experience that such controlled experimental circumstances are of vital importance in the critical evaluation of the ultimate significance of various other factors suspected of contributing to cardiovascular disease processes.

[A provocative example of comparative environmental stress, and the resulting incidence of disease, has been noted in our laboratory during studies on the incidence of mouse mammary carcinoma. Under moderate chronic but intermittant environmental stress, C3H female mice carrying the Bittner virus develop the usual 75 to 80 % mammary carcinoma by 15 months; while identical mice maintained under special environmental conditions of minimum stress have shown 0 % tumors during the same period, in current experiments in our laboratories (Riley, V., unpublished data).]

HOUSING, CARE AND STORAGE OF MICE

Protective Ventilated Shelves for Experimental Animal Storage with Minimal Stress. A simple facility has been devised for the protection of experimental animals from both external and intra-laboratory contamination. Additional important features of the facility include protection of laboratory personnel from potential hazards associated with infectious aerosols and allergenic proteins present in most animal storage and work areas. The principle of the design of this facility is that of library wall-type, or free-standing shelves back to back, enclosed by sliding or hinged plexiglass doors. Standard plastic or metal cages of appropriate size are employed in the usual way. Various types of laminar flow ventilation are feasible; however, an effective and simple arrangement presently employed is to attach an exhaust duct to one end of the shelf unit through a suitable plenum chamber, with removable filters installed at the opposite ends for the entry of clean air, usually drawn directly from the room. The contaminated air is exhausted via a window or roof fan, or other appropriate means so that air in contact with the animals never recirculates in the animal facility. Nine years experience with this system has established that it is possible to work simultaneously with a variety of viruses and other infectious entities in the same room without cross-contamination. Greatly improved health of the experimental animals has been observed, with elimination of common rodent respiratory diseases and infantile diarrhea. Experience has also shown that this housing arrangement also imposes a minimum stress environment upon the mice that are housed therein. The improved space efficiencies and odor-free characteristics of this facility permit ordinary animal rooms to be converted to biological laboratories with a more effective and convenient utilization of research space. Features and some construction details are shown in Figure 4.

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Riley, V., Spackman, D.H., and Fitzmaurice, M.A.: Critical influence of an enzyme-elevating virus upon long-term remissions of mouse leukemia following asparaginase therapy. Recent Results in Cancer Research 33:81-101 (1970).

Riley, V., Spackman, D.H., Fitzmaurice, M.A., and Lulham, C.N.: Possible mechanisms for permanent remission of leukemia in mice treated with L-asparaginase. Abstracts, Tenth International Cancer Congress, pp. 445-446 (1970).

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Spackman, D.H., Riley, V., and Teschner, L.: Asparaginase therapy in leukemic mice: Changes in plasma amino acids as modified by the LDH-virus. Abstracts, Tenth International Cancer Congress, pp. 444-445 (1970).

Santisteban, G.A., Riley, V., and Willhight, K.: Studies in virus-tumor relationships: Responses of the adrenocortical-thymolymphatic systems to the LDH-elevating virus. Abstracts, Tenth International Cancer Congress, p. 302 (1970).

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Riley, V., Spackman, D.H., and Fitzmaurice, M.A.: Influence of asparaginase and glutaminase upon free amino acids in normal and tumor-bearing mice. In: La L-Asparaginase, Boiron, M., ed., Centre National de la Recherche Scientifique, Paris, pp. 139-158 (1971).

Riley, V., Spackman, D.H., and Fitzmaurice, M.A.: Influence of the LDH-elevating virus in revealing profound alterations in host physiology during asparaginase therapy. Proc. Amer. Assoc. Cancer Res. 12:64 (1971).

Riley, V.: Rescue of an eclipsed LDH-virus by host immunosuppression techniques. Proc. Amer. Soc. Biol. Chem. (1971).

Spackman, D.H., and Riley, V.: E-N-Monomethyllysine and other plasma amino acids in leukemic mice, and effects of asparaginase. Proc. Amer. Soc. Biol. Chem., 30:1067 (1971).

Santisteban, G.A., Riley, V., and Fitzmaurice, M.A.: Thymolytic and adrenal cortical responses to the LDH-elevating virus. Proc. Soc. Exptl. Biol. and Med. 139:202-206 (1972).

Riley, V., and Spackman, D.H.: Free plasma amino acids associated with melanoma. Abstract, VIIIth International Pigment Cell Conf., Sydney, March 13-17 (1972).

Riley, V., Spackman, D.H., Fitzmaurice, M.A., Roberts, J., Holcenberg, J.S., and Dolowy, W.C.: Studies on a new glutaminase/asparaginase: Therapeutic properties, half-life, and alterations in the plasma amino acids. Proc. Amer. Assoc. Cancer Res. 13:117 (1972).

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10. FACILITIES AVAILABLE

Adequate physical facilities, composed of 16 modules of laboratory space, are available to the Department of Microbiology where these studies will be carried out. A suite of five rooms (approximately 1,000 sq. ft.) are available for experimental animal studies. This includes two animal rooms ("clean" and virus-infected) with special ventilated shelves for securely housing normal and infected mice and other experimental animals (see separate description of these facilities). Adjacent to the animal quarters are two rooms for laboratory operations. These rooms connect with either the "clean" or virus-infected animal rooms, providing an airlock and contamination barrier. An additional room is available for the safe storage and processing of food, bedding, cages, etc.

Six additional laboratories are available (approximately 2,000 sq. ft.) for carrying out the specialized research activities described in this application. A walk-in cold room, photography darkroom, and other standard laboratory facilities are available, including facilities for glass washing and sterilizing. Services of a fully equipped instrument shop are available at the adjacent Medical Center.

Available office space includes a two-module room for Departmental activities, and additional rooms for administrative, accounting, and secretarial operations. The Department provides a small reference library, including current journals that are related to cancer research.

Comprehensive laboratory facilities of a modern 500-bed hospital can also be called upon for special needs.

Two fully automated amino acid analyzers (Beckman Models 120B and 120C) equipped with digital integrating computers have been purchased for the use of the Department. These are essential instruments for studies such as proposed in this project.

Two Warburg manometric instruments, complete with calibrated glassware, are available for enzymic or other metabolic studies on normal and neoplastic cells.

A laboratory equipped for cell culture operations is a part of the Departmental facilities. Electron microscope facilities are available in the Foundation.

Following are additional items of equipment available for these studies:

Cary Recording Spectrophotometer Model 11
Cary Recording Spectrophotometer Model 15
International Refrigerated Centrifuge
Spinco Ultracentrifuge
Corning pH Meter (4 digit)
Orion pH Meter (5 digit)
Photographic Equipment
Coulter Counter, with Plotter
Incubators
Tissue Culture Inverted Microscope
Research Microscopes (several)
Zeiss Photomicroscope
Automatic Pipettes
Mettler Balances (various types)
Microtiter Equipment

11. Additional facilities required: NONE.

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Spackman, D.H., Riley, V., and Teschner, L.: Asparaginase therapy in leukemic mice: Changes in plasma amino acids as modified by the LDH-virus. Transactions, Tenth International Cancer Congress, pp. 443-446 (1970).

Riley, V., Spackman, D.H., and Fitzmaurice, M.A.: Critical influence of an enzyme-elevating virus upon long-term remissions of mouse leukemia following asparaginase therapy. Recent Results in Cancer Research 33:81-101 (1970).

Riley, V., Spackman, D.H., and Fitzmaurice, M.A.: Influence of asparaginase and glutaminase upon free amino acids in normal and tumor-bearing mice. In: La L-Asparaginase, Boiron, M., ed., Centre National de la Recherche Scientifique, Paris (1971).

Riley, V., Spackman, D.H., and Fitzmaurice, M.A.: Influence of the LDH-elevating virus in revealing profound alterations in host physiology during asparaginase therapy. Proc. Amer. Assoc. Cancer Res. 12:64 (1971) (Abstract).

Spackman, D.H., and Riley, V.: E-N-Monomethyllysine and other plasma amino acids in leukemic mice, and effects of asparaginase. Federation Proc. 30:1067 (1971) (Abstract).

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Postdoctoral Training (Research Instructor) under Dr. Thomas F. Dougherty: Cancer biology, radiobiology, biology of lymphatic tissue and studies of the lymphocytolytic effects of the adrenocortical steroid hormones, 1951 to 1953.

Lecturer in Anatomy (Elementary anatomy, gross anatomy, neuroanatomy, medical histology), University of Utah, College of Medicine, 1949 to 1951.

Teaching Assistant in Anatomy (Elementary anatomy, gross anatomy, neuroanatomy, medical histology), University of Utah, School of Medicine, 1947 to 1949.

Graduate Assistant in Zoology (Comparative vertebrate zoology and mammalian anatomy), Department of Zoology, University of Utah, 1946 to 1947.

Assistant in Zoology (General zoology and comparative vertebrate anatomy), University of Montana, Department of Zoology, 1945 to 1946.

Student Assistant in Zoology, Department of Zoology, University of Montana, 1944 to 1946.

Publications.

Santisteban, G.A.: The growth and involution of lymphatic tissue and its interrelationships to aging and to the growth of the adrenal glands and sex organs in CBA mice. Anat. Rec. 136:1 (1960).

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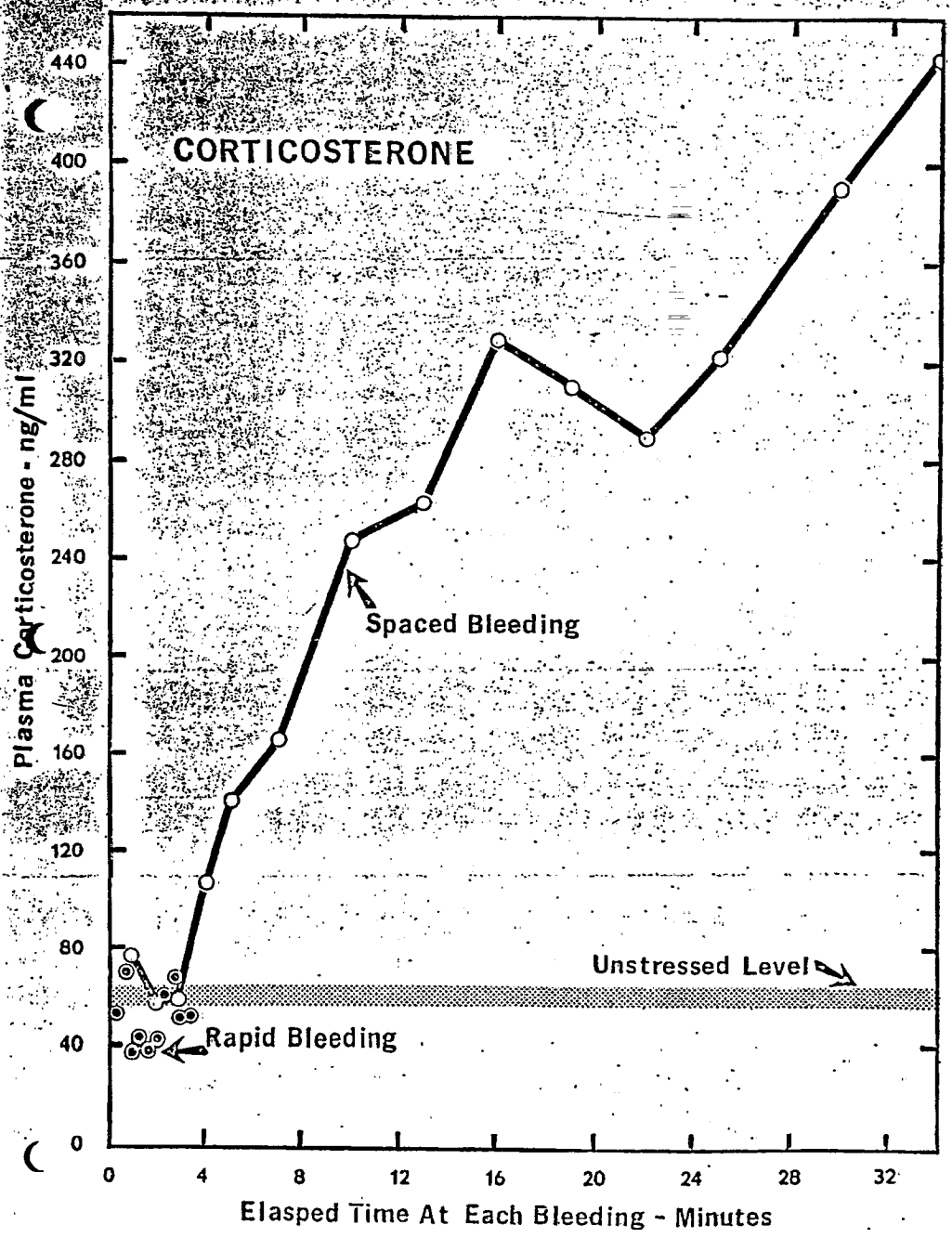

Fig. 7

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R: REDACTED MATERIAL

12. BIOGRAPHICAL INFORMATION

(Give the following information for all professional personnel listed on page 2, beginning with the Principal Investigator. Use continuation pages and follow the same general format for each person.)

NAME	TITLE	BIRTHDATE (Mo., Day, Yr.)
SPACKMAN, Darrel H.	Senior Research Biochemist	R

PLACE OF BIRTH (City, State, Country)	PRESENT NATIONALITY (If non-U.S. citizen, indicate kind of visa and expiration date)	SEX
R	R	☒ Male ☐ Female

EDUCATION *(Begin with baccalaureate training and include postdoctoral)*

INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	SCIENTIFIC FIELD
University of Utah, Salt Lake City, Utah	B.A.	1950	Chemistry
University of Utah, Salt Lake City, Utah	M.A.	1952	Biochemistry
University of Utah, Salt Lake City, Utah	Ph.D.	1954	Biochemistry

HONORS
Phi Betta Kappa
American Society of Biological Chemists
American Association for Cancer Research

MAJOR RESEARCH INTEREST	ROLE IN PROPOSED PROJECT
Amino acids, peptides and proteins involved in malignancy	Co-Principal Investigator

RESEARCH SUPPORT *(See Instructions)*

Supported by grants awarded to Dr. Vernon Riley as Principal Investigator

RESEARCH AND/OR PROFESSIONAL EXPERIENCE *(Starting with present position, list training and experience relevant to area of project, including teaching and clinical responsibilities. List all or most representative publications.*

Senior Research Biochemist, Department of Microbiology, Pacific Northwest Research Foundation, Seattle, Wash., 1968 to present.

Research Assistant Professor of Biochemistry and of Obstetrics-Gynecology, University of Washington, Seattle, Wash., 1963 to 1968.

Senior Biochemist, Spinco Division, Beckman Instruments, Inc., Palo Alto, Calif. 1959 to 1963.

Research Associate, Rockefeller Institute, New York, N.Y., 1954 to 1959.

Postgraduate Fellowship, U.S. Public Health Service, 1951 to 1954.

Publications

See following sheets.

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R: REDACTED MATERIAL

12. BIOGRAPHICAL INFORMATION

(Give the following information for all professional personnel listed on page 2, beginning with the Principal Investigator. Use continuation pages and follow the same general format for each person.)

NAME	TITLE	BIRTHDATE (Mo., Day, Yr.)
Santisteban, George Anthony	Senior Biologist	R
PLACE OF BIRTH (City, State, Country)	**PRESENT NATIONALITY** (If non-U.S. citizen, indicate kind of visa and expiration date)	**SEX**
R	R	☒ Male ☐ Female

EDUCATION: *(Begin with baccalaureate training and include postdoctoral)*

INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	SCIENTIFIC FIELD
Montana State University, Missoula, Mon.	B.A. (Honors)	1945	Zoology
University of Utah, Salt Lake City, Utah	M.A.	1949	Zoology
University of Utah, Salt Lake City, Utah	Ph.D.	1951	Anatomy
University of Utah, Salt Lake City, Utah	Postdoctoral	1951-53	

HONORS

American Association for Cancer Research, American Association of Anatomists, Phi Sigma Biological Honorary Society, and New York Academy of Sciences

MAJOR RESEARCH INTEREST	ROLE IN PROPOSED PROJECT
Stress, adrenocortical-thymus function and malignancy.	Co-Principal Investigator and Collaborator

RESEARCH SUPPORT *(See instructions)*

$124,089; NIH, USPHS, 09758-05;

Social Stimulation, RNA, and Cardiovascular Disease, 09-01-72 to 08-31-75.

RESEARCH AND/OR PROFESSIONAL EXPERIENCE *(Starting with present position, list training and experience relevant to area of project, including teaching and clinical responsibilities. List all or most representative publications.*

Affiliate, Fred Hutchinson Cancer Research Center, Seattle, Washington, 1973 to present.

Senior Biologist, Pacific Northwest Research Foundation, Seattle, Washington, 1968 to present.

Associate Professor of Biology, Seattle University, Seattle, Washington, 1968 to present.

Acting Head, Department of Biology, Seattle University, Seattle, Washington, 1966 to 1968.

Associate Professor of Biology, Seattle University, Seattle, Washington, 1964 to 1968.

Special Fellowship, U.S.P.H.S. to continue training and do research at the University of Gothenburg (Goteborg), Sweden, under sponsorship of Professor Holger Hyden, 1966 to 1967.

Research Associate at University of Gothenburg, Sweden, to obtain special training in the microanalytical methods for studying intracellular RNA, under sponsorship of Professor Holger Hyden. (On leave from University of Southern California, Research Associate), 1963 to 1964.

Assistant Professor of Physiology (Neurophysiology), University of Southern California School of Medicine, 1959 to 1963.

Assistant Professor of Anatomy (Neuroanatomy, Microscopic Anatomy), University of Southern California, 1954 to 1959.

Assistant Professor (Gross Anatomy), Medical College of Virginia, Richmond, Virginia, 1953 to 1954.

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